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Full Name of Author — Nom complet de l'auteur

LEWIS L. TOMALTY

Date of Birth — Date de naissance

SEPT 2/56

Country of Birth — Lieu de naissance

CANADA

Permanent Address — Résidence fixe

292 PARK AVENUE
BROWNSBURG, QUEBEC
JOV 1A0

Title of Thesis — Titre de la thèse

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DR. F. L. JACKSON & MISS Y. E GOODMAN

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Serum and Local Immune Responses, Bacteriology, and Transmissibility of Experimental Respiratory Tract *Bordetella pertussis* Infection in Mice

by

© Lewis L. Tomalty

A THESIS

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(SIGNED) Lewis Tomalty

PERMANENT ADDRESS:

292 Park Avenue

Brownsburg, Quebec

DATED April 15 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Serum and Local Immune Responses, Bacteriology, and Transmissibility of Experimental Respiratory Tract *Bordetella pertussis* Infection in Mice submitted by Lewis L. Tomalty in partial fulfilment of the requirements for the degree of Master of Science in Medical Bacteriology.

J. Goodman F.H. Jadwin

Supervisors

James Brunton

G. Pahl

Date 31 March 1981

Abstract

Mice intranasally inoculated with a nonlethal dose of *Bordetella pertussis* were examined for 177 days. Infection was determined by culture and direct immunofluorescence and a double antibody indirect Enzyme-linked Immunosorbent Assay (ELISA) was employed for studying pertussis antibody responses in serum and respiratory tract secretions.

A high incidence of *B. pertussis* recovery in culture persisted for 21 days and thereafter occasional samples yielded positive cultures until day 44 after infection. Although the majority of cultures taken after the third week of infection were negative, organisms were detectable by direct immunofluorescence from several mice at each sample date until day 129.

Increases were observed in the serum antibody levels of the five immunoglobulins tested (IgG1, IgG2a, IgG2b, IgM and IgA). Pertussis specific antibodies were detectable 16 to 18 days after infection and were still found in high levels 177 days after infection. Peak IgG levels were attained by approximately the 7th to 8th week of disease, whereas maximal IgM levels were observed between the 3rd to 7th weeks. IgA appeared in parallel with the other immunoglobulins, but it could not be determined if a maximal level of serum IgA had been attained when the study was completed at 177 days.

Pertussis IgA and IgG (subclasses G1, G2a, G2b) were detected in mouse tracheobronchial washes but IgM was not

detected. IgA was present at day 18, peaked by about day 30 and was still present in high levels at day 177. IgG was present in much lower levels than IgA in the washes and on several sample dates returned to negative values after a low peak. The strong and early appearance of secretory IgA makes it a possible indicator of infection in retrospective confirmation of a diagnosis of pertussis.

Exposure of uninoculated mice to infected mice indicated that although *B. pertussis* cultures were largely negative after the third week of infection, the inoculated mice were still capable of transmitting *B. pertussis*. The organism could not be cultured from exposed, uninoculated mice but direct immunofluorescence and antibody responses indicated infection in these mice. This observation provides further evidence for the possibility of a carrier state.

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I. Introduction

Pertussis, a disease with many intriguing aspects, remains common in spite of widespread immunization. The organism responsible produces a variety of unusual biological effects in experimental animals, yet the pathogenesis of the human disease is not yet clear nor has the protective antigen(s) been identified with certainty. There are gaps in our knowledge of the epidemiology of the disease and the immunological response, and for reasons not entirely clear, the laboratory confirmation of *B. pertussis* infections by culture is often unsuccessful.

The first written accounts of whooping cough appeared in the 16th century. The disease was epidemic in Europe by the 1700's and continued as a prominent malady until the advent of effective vaccines in the early 1950's (Gordon and Hood, 1951; Kuronen and Huovila, 1978). Such vaccines have greatly lowered the incidence and severity of clinical disease but infections are still not uncommon (Varughese and Acres, 1979).

Bordet is credited with first describing the organism and his name remains associated with the genus. In 1906, Bordet and Gengou isolated *B. pertussis* on a solid medium containing glycerinated potato extract and 50% blood (Bordet and Gengou, 1906; Rowat, 1957), the basis for the medium still used today.

The genus *Bordetella* consists of three species: *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* . *B.*

pertussis is the usual etiologic agent of pertussis but *B. parapertussis* is occasionally recovered from patients afflicted with the pertussis syndrome. In the United States and Europe, *B. parapertussis* is estimated to account for 5% of pertussis infections (Linnemann and Perry, 1977).

B. bronchiseptica is primarily an animal pathogen (Goodnow, 1980) afflicting rodents, dogs, cats, swine, and numerous other species. Humans are not usually considered as natural hosts for *B. bronchiseptica*. There have, however, been isolated instances of human infection (Ghosh and Tranter, 1979; Goodnow, 1980).

B.pertussis: The Organism

B. pertussis is a small, aerobic, non motile, Gram negative coccobacillus. It is a delicate organism very susceptible to drying and to inhibitory substances in the culture media. Growth requirements are relatively simple but the addition of blood, charcoal, starch or ion exchange resins to the media is necessary to counteract inhibitors (particularly fatty acids) (Field and Parker, 1979).

B. pertussis has the tendency to undergo phase variation in the laboratory from a smooth, virulent form (phase I) to a rough form (phase IV) which is nonvirulent and usually grows on nutrient agar (Munoz and Bergman, 1977). Intermediate phases II and III have also been described. Metabolic, morphologic and antigenic changes accompany the transformation from phase I to phase IV forms. The smooth forms agglutinate in antisera to Phase I

organisms and have the ability to induce active immunity and to sensitize mice to various agents. The rough cultures do not specifically agglutinate in antisera to Phase I forms and lack the ability to protect mice from infection or to sensitize them.

A capsule has been observed on *B. pertussis* but it is not a prominent feature of most strains and is not considered to be antiphagocytic (Olsen, 1975).

Electron microscopy has shown surface projections on phase I organisms (Morse and Morse, 1970) which have been described as a form of pili or fimbriae. Filamentous hemagglutinin obtained from culture supernatants of phase I *B. pertussis* is believed to be derived from the fimbriae on the bacterial surface. Electron microscope studies showed that virulent, hemagglutinating phase I pertussis adhered to mammalian cell membranes whereas nonvirulent, nonhemagglutinating phase III bacteria did not (Sato et al, 1978).

Agglutinogens, heat labile protein antigens (K antigens), are associated with the cell envelope of *B. pertussis* and form the basis for a serotyping scheme. Phase I *B. pertussis* strains contain one or more heat labile K antigens of which there are up to 8. K antigenic factor 1 is present in all strains but other K antigens differ between strains and thereby form the serotyping scheme (Andersen, 1953).

Certain of the pathologic effects of *B. pertussis* are

felt to be caused by the heat-labile (dermonecrotic) toxin (Olsen, 1975). This toxin, of cytoplasmic origin, probably contributes significantly to pathogenesis by paralysing cilia, which in turn, leads to impaired defense mechanisms.

An endotoxin of *B.pertussis* has been purified and has the typical properties of lipopolysaccharides from other Gram-negative bacteria. It is doubtful that this toxin plays an important role in pathogenesis although it may cause some adverse effects after immunization (Olsen, 1975).

In addition to the components of *B. pertussis* described here, the supernatant fluids of phase 1 cultures produce a variety of biological effects following injection into experimental animals (Table 1).

Table 1

Biological Effects of Supernatant Fluid of Phase I *B. pertussis* Cultures:

Histamine-sensitization
Lymphocytosis
Beta-adrenergic blockade-like effect
Late appearing toxicity
Islet cell stimulation
Adjuvant effect
Mitogenicity

Recently Morse (1976) has isolated an apparently pure protein from culture supernatants (molecular weight 67,000 to 73,000) which produces lymphocytosis, histamine sensitization and other effects. This suggests that one component-designated "Pertussigen" by Munoz (Munoz and Bergman, 1977;1978) or "Pertussis toxin" by Pittman (1979), is responsible for the variety of biological effects.

Epidemiology

The epidemiology of pertussis is considered quite straightforward, as man is the only known reservoir of the organism (Linnemann, 1978). *B. pertussis* is transmitted primarily by droplets, and pertussis is one of the most contagious of diseases. Attack rates in a susceptible population can be as high as 100% (Lambert, 1965).

There are no proven chronic carriers of phase I *B. pertussis*. For example, Linnemann et al (1968) found no asymptomatic carriers among 1102 individuals (including well babies, preschool and school children, and family and neighborhood contacts of cases) he investigated during a pertussis epidemic. However, there is the possibility that carriers do exist, but carry the organisms in a form other than phase I (Linnemann et al, 1968). Phase IV organisms differ substantially from phase I in cultural characteristics and morphology and thus they may go unrecognized in culture. Altered states of *B. pertussis* such as spheroplasts have also been identified (Mason, 1966), and it is conceivable that other forms of the bacteria exist.

In the prevaccine era, the highest incidence of pertussis was in the 4-6 year old group (Linnemann, 1978). With the introduction of vaccines, major alterations in epidemiology patterns have occurred. There is an increased frequency of adult disease and the appearance of milder, atypical pertussis (Linnemann and Nasenbeny, 1977; Linnemann, 1978; Nelson, 1978). Consequently, the reservoir of infection may have changed from the child with typical disease to either the child or the adult with atypical disease.

Clinical Disease

The term "Pertussis" has largely replaced "Whooping Cough", as *B. pertussis* produces an array of clinical manifestations which range from mild respiratory tract infections to severe cases of typical whooping cough (Linnemann and Nasenbeny, 1977). The disease still presents a serious health hazard particularly in the very young who expierence both the highest morbidity and mortality (Olsen, 1975). For example, in one outbreak, 5 of 21 positive cases required hospitalization and 2 of these children were critically ill (Field and Parker, 1977).

Following an incubation period of approximately 10 days, pertussis characteristically progresses through three stages: catarrhal, paroxysmal, and convalescent (Pittman, 1970; Olsen, 1975; Munoz and Bergman, 1977; Linnemann, 1978). The catarrhal stage lasts from 1-2 weeks and is normally indistinguishable from an upper respiratory tract

infection although coughing becomes increasingly severe towards the end of the phase. Recovery of bacteria is highest in this acute phase, but declines rapidly with the onset of the paroxysmal stage. Paroxysms of coughing, followed by the inspiratory whoop, dominate this phase. Although bacterial cultures become negative early in the paroxysmal stage, coughing may last 4-8 weeks, and continue in the convalescent stage for weeks or months before the patient finally recovers. If a patient is treated with an appropriate antibiotic, such as erythromycin, the culture becomes negative within a few days, but unless treatment is started in the catarrhal stage, coughing will continue unchanged (Linnemann, 1978).

One of the diagnostic criteria of whooping cough is a relative lymphocytosis which appears with the paroxysmal coughing and declines with the disappearance of *B. pertussis* but may persist for two to three months (Pittman, 1970).

The most common complications of pertussis are pulmonary superinfections. Organisms such as *Haemophilus influenzae*, staphylococci, pneumococci, and B-hemolytic streptococci, are the usual offenders (Jamieson, 1973).

The central nervous system may occasionally be affected in pertussis (Olsen, 1975; Linnemann, 1978). The most common neurologic complication is convulsion, but paralysis, coma, blindness, deafness and movement disorders have also been reported. These conditions probably result from anoxia and occasionally hemorrhage although hypoglycemia has also been

implicated in central nervous system disorders (Linnemann, 1978).

Laboratory Diagnosis

The most important factors in successful isolation of *B. pertussis* are the collection of suitable specimens, their rapid processing and the use of freshly prepared, moist plating media (Parker and Linnemann, 1980). Routine cultures should be performed by collecting pernasal swabs or nasopharyngeal secretions. Direct plating is the most reliable culture technique but when not possible, a special transport medium may be used (eg. Regan and Lowe, 1977). Although isolation of organisms is the primary method of confirming disease, culture is often unsuccessful. Most studies report recovery rates of less than 50% (Linnemann, 1978). Infection by a number of agents other than *B. pertussis* (ie. *B. parapertussis*, *B. bronchiseptica*, viruses) (Olsen, 1975; Linnemann, 1978), can produce symptoms suggestive of pertussis, and thereby compound diagnostic difficulties.

Given that recovery of organisms is not always reliable, alternate methods of diagnosis have been sought. Direct immunofluorescent staining of nasopharyngeal smears (Donaldson and Whitaker, 1960) has become the most popular rapid diagnostic method. However, without confirmatory cultures, problems of interpretation limit this technique (Broome et al, 1978), but it can be useful for rapid presumptive diagnosis.

The use of serological methods (Olsen, 1975) may aid in confirming pertussis but generally such techniques may be of help only retrospectively and are unreliable for diagnosis.

Improvements in pertussis diagnostic techniques are therefore still required, particularly for confirming culture negative cases.

Pathogenesis

In human and experimental disease, *B. pertussis* produces a localized superficial infection of the respiratory tract. Collier et al (1977), using hamster tracheal organ cultures showed that *B. pertussis* preferentially attaches to the ciliated cells. This occurence produced ciliostasis and a marked destruction of the subcellular organelles followed by expulsion of these cells from the epithelial layer. There was no evidence for either inter or intracellular invasion. Nonciliated cells appeared to be unaffected, a fact later confirmed by Muse et al (1977) using the same model, and studied by scanning electron microscopy. Matsuyama (1977) also demonstrated the attachment of *B. pertussis* to ciliated epithelial cells in cultures of rabbit tracheal mucous membranes. He showed that virulent phase I organisms adhered to the site of inoculation whereas avirulent phase III organisms were moved up the trachea by ciliary action, thereby indicating the importance of attachment in infection.

The mechanism by which attachment of *B. pertussis* to the ciliated epithelia appears to be accomplished (at least

in part) is by the bacterial fimbriae or hemagglutinin (Sato et al, 1978). The subsequent ciliostasis is most likely an effect of the pertussis heat labile toxin (Olsen, 1975). Ciliostasis would result in major interferences with the normal clearing mechanisms of the respiratory tract. This, coupled with increased mucus secretions and extensive inflammation would encourage further multiplication of organisms.

The most obvious symptom of pertussis is the paroxysmal cough which often persists for much longer than the acute stage of the disease. The mechanism of cough production remains unknown. Direct injury of the ciliated epithelia would have some effect but prolonged coughing is believed to be related primarily to an altered Beta-adrenergic system (Linnemann, 1978; Munoz and Bergman, 1978).

It appears obvious that certain other of the clinical manifestations of the disease are due to the biological components as listed in Table 1. Indications are that a single protein component may induce in animals various biological reactions occurring as a result of a single underlying mechanism (Morse, 1976). Pittman (1979) feels that this substance (designated "pertussis toxin" , or "pertussigen" Munoz and Bergman, 1977;1978)) is the cause of the harmful effects and also the prolonged immunity in pertussis.

Experimental Infections and Immunology

B. pertussis is not a natural pathogen for animals.

However, a number of species have successfully been infected (Pittman, 1970). The first successful animal infection was accomplished when Burnet and Timmins (1937) produced pertussis respiratory infections in mice. Since then, this animal model has been studied extensively. Three routes of infection have been used: intraperitoneal, intracerebral, and intranasal. Organisms injected by the intraperitoneal route cause death in mice in 1-3 days. However, a large dose is required (2×10^9 bacteria) and death is due to toxemia rather than infection (Pittman, 1970).

Following an intracerebral injection, the bacteria multiply on the ventricle wall in association with the ciliated ependymal cells, a site very similar to the ciliated epithelium of the human respiratory tract (Pittman, 1970; Hopewell et al, 1972), until lethal numbers of bacteria are reached. The intracerebral route is used primarily in vaccine potency assays. In the immunized mouse, the organisms multiply in the brain for 4-6 days and then rapidly disappear. The intracerebral challenge in potency assays for pertussis vaccines provides relative values which correlate with the efficacy of vaccines in humans (Medical Research Council, 1959).

Two types of challenge by the intranasal route have been used by different workers, lethal and sublethal. When a lethal dose (10^7-10^8 organisms) is given to mice, the bacteria grow for several days until a critical level, at which the mouse dies (Dolby et al, 1961). Sublethally

infected mice (10^6 organisms or less, Burnet and Timmins, 1937; Dolby et al, 1961) resemble human pertussis infections in persistance of bacteria and immune response (Geller and Pittman, 1973) as well as pathophysiological reactions (Pittman, 1980). In sublethally infected mice, the bacteria multiply for 7-14 days, followed by a gradual decline. However, it is possible for infections to last 3-4 weeks (Pittman, 1970). Histamine sensitization in the intranasally infected mouse develops by day 15-20 and persists until at least day 50 (Geller and Pittman, 1973) which parallels paroxysmal coughing in the child. A marked leukocytosis also develops in intranasally infected mice. After recovery from a lung infection, there is immunity to further intranasal challenges (Pittman, 1970).

A thorough investigation of the sequence of events in murine pulmonary pertussis has been carried out by Cheers and Gray (Gray and Cheers, 1967; Cheers, 1969; Cheers and Gray, 1969; Gray and Cheers, 1969). In the pre-immune phase of infection, there is an initial logarithmic increase in the numbers of bacilli. Following this period of immunological incompetence, the bacteria are reduced by leukocyte invasion to approximately 1% of the pre-immune population. During this period, cellular immune processes appear to be the predominant control mechanism as there is a shift in the bacterial habitat from an extra to an intracellular phase. Although now found within the alveolar macrophages, the organisms are able to survive this

intracellular environment. The net effect is a steady state known as "complaisance" in which the numbers of bacteria are strictly controlled. Any alteration in the numbers of bacteria either by chemotherapeutic reduction or by superinfection is rapidly corrected to the original. This phase appears to last for 5-6 weeks at which time culturable organisms are eliminated, probably by protective humoral factors. For example, North (1946) found that serum from intranasally infected mice bled on days 30-50 passively protected mice, whereas 14-day serum was ineffective.

During the complaisant phase, the alveolar macrophages exhibit increased killing power although they maintain normal phagocytic capacity. With the appearance of the final bactericidal phase, the macrophage's enhanced killing power declines. It is interesting to note that no delayed type hypersensitivity could be demonstrated in spite of the apparent cell-mediated immune reaction (Cheers, 1969).

Indications are that final elimination of bacteria requires humoral antibody, although cell-mediated immunity is involved, at least in mice. (Gray and Cheers, 1969)

In human pertussis, reinfection after an acute *B. pertussis* infection is uncommon, however second attacks may be mild and thus not diagnosed. A degree of resistance to infection is also conferred after parenteral inoculation of killed organisms. This suggests that serum antibody has a role in protection against human pertussis. However, the extent and duration is uncertain. Immunity is not life long

as was illustrated in a pertussis epidemic in Michigan. In those persons immunized within less than 1 year, there was an attack rate of 20%, but in those where the interval since immunization had been over 10 years, the attack rate was 58%. Second attacks of pertussis were also noted during the epidemic (Lambert, 1965).

Serum antibody responses have been measured by a variety of techniques, including complement fixation (Bradstreet et al, 1972), hemagglutination inhibition (Dolby and Stephens, 1973), agglutination (Dolby and Dolby, 1969), bactericidal activity (Aftandelians and Connor, 1973), precipitation (Aftandelians and Connor, 1973a), mouse protection (Dolby, 1972), and immunofluorescence (Geller and Pittman, 1973). However, resistance to infection does not correlate well with antibody detected by any one of these methods.

The basic pattern of antibody response is illustrated in Donald's study (1939) detecting complement fixing antibodies. Antibodies began to appear in the second to third week after infection but did not reach a peak until the seventh to eighth week. It was observed that the appearance of antibodies correlated with the disappearance of organisms in culture, suggesting a role for serum antibodies in recovery from infection.

Despite the evidence for the presence of antibodies, the mechanism by which they function is still unknown. One of the basic problems in solving this has been in attempting

to correlate immune reactions in serum with resistance to superficial infection of the respiratory tract. It would appear that immunity in pertussis is governed by both serum and local (secretory) immune responses.

Pittman (1979), has postulated that the local secretory antibody response is the first line of defense against infection. In this process, the antibodies are directed against the fimbrial hemagglutinin thereby inhibiting attachment of the organisms to the host cilia. Serum antibody against "pertussis toxin", is suggested, as the second line of defense with this long lasting response being directed against those biological components which cause the harmful effects associated with the disease.

In spite of the potential importance of the local immune response in pertussis, relatively little is known about this mechanism. Holt (1972) detected antibodies that interfered with the adhesion of *B. pertussis* in the saliva of a Taiwan monkey after aerosol immunization. Thomas (1975), using a sensitive radioimmunoassay technique observed increased levels of antibodies in human respiratory secretions after aerosol immunization with pertussis vaccine. Geller and Pittman (1973) followed the level of immunoglobulins in serum and tracheobronchial wash after infecting mice with *B. pertussis*. They reported the presence of both IgA and IgG specifically bound to *B. pertussis* in mouse tracheobronchial washes by day 15 after infection. Recently, Goodman et al (1981) detected pertussis specific

IgA in nasopharyngeal secretions of children with pertussis. An enzyme-linked immunosorbent assay (ELISA),was used to detect the antibodies which were present by the second to third week of illness. These workers suggested that the presence of pertussis specific IgA in secretions could be used as an indicator of recent infection.

There is a need for more precise information about the secretory and serum antibody responses during the course of pertussis infection. The purpose of this study was therefore to follow these responses using the mouse respiratory infection model. Mice were sublethally infected with *B. pertussis* by intranasal instillation and samples of tracheobronchial wash and serum collected at intervals. The presence of *B. pertussis* was detected by culture and immunofluorescence and levels of pertussis specific antibodies determined by ELISA. This technique allowed for the determination of the immunoglobulin class of specific antibody and because of its high level of sensitivity could be used on very small samples.

During the progress of this study it was noted that *B. pertussis* could be detected in tracheobronchial wash by immunofluorescence for several months after cultures became negative. A second experiment was therefore designed to determine if mice at this "carrier" stage were still capable of transmitting infection.

II. Materials and Methods

A. Experimental Design

Experiment 1

Intranasal inoculations with *B. pertussis* (strain X376) were performed on 120 mice. The mice were housed 5 per cage and given unlimited food and water. Control, uninoculated mice were housed in the same manner but in a separate room. The course of the disease was followed by two methods:1. Culture and direct immunofluorescent staining (FA) 2. Serum and local immune responses to *B. pertussis* as determined by a double antibody indirect ELISA. Five infected mice were sacrificed at approximately 4 day intervals for the first 8 weeks and thereafter at weekly intervals for a further 10 weeks. The final followup group of mice was examined at approximately 25 weeks. Two control mice were sacrificed with every second group of infected mice.

Experiment 2

Mice were intranasally inoculated with *B. pertussis* (strain X376) on day 1 and separated to 3 per cage. At subsequent weekly intervals, (to 9 weeks), 2 uninoculated mice were added to each cage and the cages placed in groups. There were 3 groups; 10, 20, and 30 days after exposure and it was on these days that mice were sacrificed. For the 7, 14, and 21 day periods, each group consisted of 2 cages (total of 4 exposed mice, and 6 infected mice per group) whereas the remainder of the time periods (28,35, 42, 49,

56, and 63), each group consisted of 3 cages (total of 6 exposed mice and 9 infected mice per group).

As an example, mouse 'x' was placed in a cage containing mice infected with pertussis 14 days previously. Then 20 days later, the exposed mouse (mouse' x') was sacrificed.

Samples of TBW taken from sacrificed (exposed) mice were cultured, examined by direct immunofluorescence for *B. pertussis* and tested for secretory IgA by ELISA. Serum samples were tested by the ELISA for the presence of pertussis IgG1, IgG2a, IgG2b, and IgM. as in Experiment 1.

B. Media

Charcoal Horse Blood Agar (CHBA)

(Sutcliffe, 1972)

Oxoid charcoal agar (Oxoid CM 119) plus 1% Difco proteose peptone no.3 and 10% horse blood. The medium was used within a week after preparation and did not contain added antibiotics.

Charcoal Enrichment Media

(Regan and Lowe, 1977)

One half strength Oxoid Charcoal Agar with a final concentration of 10% horse blood, dispensed in bijoux bottles and used within 2 weeks after preparation.

C. Bacterial Strains

B. pertussis (X376)

A clinical isolate of *B. pertussis* (X376), found in preliminary experiments to be pathogenic for mice was employed throughout the study for mouse inoculations. The strain was preserved by lyophilization and a vial of freeze dried culture was revitalized and subcultured no more than twice before inoculating mice. This strain contains serofactors 1,3 and 6 (determined by M. Magus, Special Reference Bacteriology, Ontario Ministry of Health, Toronto).

B. pertussis (strain 1494)

Obtained from D.W.Stainer,Connaught Laboratories, Toronto, was used for the preparation of the ELISA antigen and contains serofactors 1, 2, and 3.(Sekla et al, 1978).

B. pertussis (strain H36)

Obtained from B.W.Lacy, Westminster Hospital Medical School, London, England, was used as the positive control strain for the direct immunofluorescent techniques.

B. parapertussis (strain D42875)

A clinical isolate of *B.parapertussis* from the Provincial Laboratory of Public Health, Edmonton, was used as the negative control for direct immunofluorescence.

Staphylococcus aureus (strain Cowan 1)

S. aureus was used for Protein A production.

D. Mice

Six week old female Swiss ICR mice were obtained from the breeding colony of the Provincial Laboratory of Public Health, Edmonton.

E. Intranasal Inoculations

Mouse intranasal inoculations with *B. pertussis* were performed essentially as described by Burnet and Timmins (1937). The 48 hour growth from one charcoal horse blood agar (CHBA) plate was carefully suspended by vortexing in 1% weight/volume (w/v) Difco-Casamino Acids to an Absorbance at 600nm (A 600nm) (Unicam SP1800 Ultraviolet Spectrophotometer) of 0.1. A 1/25 dilution of this suspension was made and one drop from a pasteur pipette (approximately 0.025ml) contained approximately 2.5×10^5 colony forming units (CFU), as determined by the Miles and Misra colony count technique. This was the required inoculum to produce sublethal infections in mice (Burnet and Timmins, 1937; Geller and Pittman, 1973). Suspensions were prepared immediately before inoculating to ensure as uniform results as possible.

Mice were anaesthetized in a glass jar containing cotton balls soaked with ether. When completely anaesthetized, and breathing rapidly but not gasping, the mouse was removed from the jar and held horizontally. One drop of the bacterial suspension was then placed on the external nares. Only if the complete inoculum was rapidly

drawn in without bubbling was the mouse included in the experiment.

F. Sample Collection

Tracheobronchial Wash (TBW)

After anaesthetizing the mouse with sodium pentobarbitol (nembutol), the trachea was exposed and a fine hole made with scissors immediately below the pharynx. Fine gauge polyethylene tubing (PE 10-Intradermic Polyethylene Tubing, Clay Adams, Parsippany, N.J.) attached to a 27 gauge needle was inserted 3-4mm into the trachea and sutured in place. A tuberculin syringe containing 1.0ml of 0.85% (w/v) sodium chloride was attached to the needle. The tracheobronchial tree was washed by slowly injecting and withdrawing the saline 3 to 4 times. Gentle pressure exerted on the thorax ensured that the maximal amount of wash could be retrieved(about 0.6ml).

Samples were cultured for *B. pertussis* immediately after collection, smears were prepared for direct immunofluorescence staining, and the remainder of the five TBW's were pooled and stored at -20°C.

Sera

After taking the TBW's, the mice were bled by cardiac puncture of the left ventricle. Blood was centrifuged for 1 minute in an Eppendorf Microcentrifuge (Model no. 5412) and the serum removed and stored individually at -20°C.

Trachea and Lung

Approximately 1 cm of trachea was removed from the mouse and opened for culturing. The left lung was also removed and cut open to culture. Both the trachea and lung were smeared directly onto CHBA plates and left in place.

G. Detection of B. pertussis in Samples

Culture

Specimens were cultured for *B. pertussis* on freshly prepared HBCA plates without added antibiotics. Cultures were incubated at 36°C for 3 to 5 days in sealed jars containing a vial of water to maintain adequate humidity.

Specimens obtained from mice for culture included a drop of TBW, a portion of opened trachea, and a cut surface of a lung lobe. These were all plated directly and incubated for 5 days before recording as negative. Calcium alginate swabs of TBW were also stabbed into charcoal enrichment media. These were incubated 48 hours and then subcultured to CHBA plates. *B. pertussis* was detected by typical colonial morphology and the identification confirmed by direct immunofluorescence.

B. pertussis, strain X376 had previously been tested to ensure that it retained viability in 0.85% saline during the time period of exposure in TBW before plating. Organisms were suspended in saline and counts done by the Miles and Misra technique at time intervals up to 90 minutes. No significant change in CFU's was noted.

Immunofluorescent Staining for B. pertussis

Fluorescein conjugated *B. pertussis* antiserum, obtained from Difco Laboratories (Bacto-FA *B. pertussis* antiserum) was used in the direct immunofluorescent (FA) test to detect *B. pertussis*. The method followed was as per instructions provided with the product. The conjugate was titrated using *B. pertussis*, strain H36. This strain also served as the positive control in the test and *B. parapertussis* strain D42875 was used as the negative control. All mouse TBW's were smeared on a glass slide (Clay Adams Fluorescent Antibody Slides), air dried, and heat fixed. A drop of fluorescein conjugate was spread over the surface of each smear, and the slides were then incubated in a moist chamber for 30 minutes at room temperature. Next were two 5 minute washes in FTA Hemagglutination Buffer (Appendix 1) (BBL Laboratories) followed by two 5 minute washes in distilled water. After air drying, the slides were mounted with Bacto-FA Mounting Fluid (Difco). Slides were scanned the same day on a Zeiss Jena Fluoval Fluorescent Microscope (with a dark field condenser and excitation filters UG1, BG12) for the presence of fluorescent organisms of typical or atypical morphology. Negative results were not recorded until the slides had been scanned for approximately 10 minutes.

H. Indirect ELISA

Antigen Preparation

The antigen for the ELISA was prepared from *B. pertussis* vaccine strain 1494, by the method of Goodman et al (1981). The 48 hour growth from CHBA plates was suspended in PBS and washed twice. The pellet was resuspended in approximately 10ml of carbonate-bicarbonate buffer (Appendix 1). The suspension was placed in an ice bath and sonicated by a Biosonic III (Bronwill Scientific, Rochester, N.Y.) with a 3/8 inch probe at 180 output in 60 second pulses, until obvious lysis of the cells had occurred. The suspension was then centrifuged at 15,000rpm (Beckman SS34 Rotor) for 30 minutes and the clear, slightly yellow supernatant collected. The protein content was determined by the Bio-Rad Protein Assay (a dye binding protein assay) (Bio Rad Laboratories, Richmond, Ca.), and the antigen stored in aliquots at -70°C until use.

Anti-mouse Immunoglobulins

Rabbit antimouse:IgM (lot 29), IgA (lot 25), IgG1 (lot 20), IgG2a (lot 21), IgG2b (lot15) were obtained from Miles Laboratories (Elkhart, Indiana).

Conjugates

i. Horse Radish Peroxidase (HRPO)

Rabbit anti-mouse immunoglobulin classes A, M, G (subclasses G1, G2a, G2b) conjugated with HRPO were prepared essentially by the technique of NaKane and Kawaoi (1974). To 5.0mg of HRPO (Sigma Type vi) dissolved in 1.0ml of 0.3M sodium

carbonate (pH 8.1) was added 0.1ml of a 1% volume/volume (v/v) solution of 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N.Y.) in absolute ethanol for 1 hour at room temperature. A 1.0ml amount of aqueous 0.08M sodium periodate was added and mixed for 30 minutes at room temperature. Then, 1.0ml of 0.16M ethylene glycol in distilled water was added and mixed for 1 hour at room temperature. The resulting solution was dialyzed at 4°C for one day with several changes of 0.01M sodium carbonate buffer, pH 9.5. The dialysate was gently mixed with 5.0mg of antimouse immunoglobulin for 3 hours at room temperature, followed by the addition of 5.0mg sodium borohydride. The solution was left for 3 hours at 4°C before dialyzing against Phosphate Buffered Saline (PBS) pH 7.2 (Appendix 1) for 24 hours with four-1 liter changes of buffer. The conjugated material was separated from the unconjugated protein and enzyme by filtration on an 85 X 1.5cm Sephadex G-200 (Pharmacia, Uppsala. Sweden) column, equilibrated with PBS pH 7.2. The peaks that showed optical density activity at both 280nm (for protein) and 403nm (for peroxidase) were pooled and concentrated by ultrafiltration with an XM 100 membrane filter (Amicon, Lexington, Mass.) to a 2ml volume and stored in the dark at 4°C.

Two alterations to the above method were subsequently made (Nakane, 1979), omission of the addition of sodium borohydride and doubling the amount of immunoglobulin.

ii. Alkaline Phosphatase

Alkaline phosphatase (Calf intestine, type vii; Sigma) was covalently linked to rabbit antimouse immunoglobulin classes A, M, and G (subclasses G1, G2a, G2b) essentially by the procedure of Voller et al (1976). The enzyme suspension was centrifuged in an Eppendorf microcentrifuge (model no. 5412) for 2 minutes and the supernatant discarded. Five mg of enzyme were added to 2.0mg of immunoglobulin in 1.0ml of PBS and mixed at room temperature. The resulting solution was dialyzed extensively against PBS at 4°C (36 hours with six-1 liter changes of buffer). 70% glutaraldehyde (Ladd Research Industries, Burlington, Vt.), opened immediately before use was added to a final concentration of 0.2%. After incubation for 2 hours at room temperature, the solution was again dialyzed against PBS (36 hours with six-1 liter changes of buffer). The dialysis tube was transferred to 0.05M trishydroxymethylaminomethane (TRIS) buffer pH 8.0 (Appendix 1) and dialysis was continued for a further 24 hours with four-1 liter buffer changes. Conjugated material was separated from unlabelled enzyme and free immunoglobulin by filtration on an 85 X 1.5cm Sephacryl S300 (Pharmacia) column equilibrated with 0.05M TRIS pH 8.0. Peak fractions were pooled and concentrated to a 2ml volume by ultrafiltration using an XM 100 membrane filter (Amicon) and stored in the dark at 4°C.

Indirect ELISA Protocol

A chequerboard titration was carried out to find the

optimal concentrations of the antigen and sera for use in the indirect ELISA. Serial dilutions of the antigen were made in coating buffer (Appendix 1) and sensitized to the plates. Three reference sera (positive, weakly positive and negative) were serially diluted and added to the wells so that each dilution of serum was reacted with all the antigen dilutions. The optimal dilutions were the highest dilutions which gave the maximal reactivity with the positive serum and minimal reactivity with the negative serum.

In essence, the ELISA was performed according to the method of Voller et al (1976). The wells of flatbottom, polystyrene microtiter plates (Dynatech Laboratories Inc., Alexandria, Va.) were sensitized with 0.2ml of antigen diluted in coating buffer (5ug/ml) by incubating at 4°C overnight in a humid container. All subsequent steps were carried out at room temperature with incubations in a moist container. The plates were then washed three times with Phosphate Buffered Saline-Tween (PBS-T). The washing step involved flooding the plates with PBS-T, agitating for 3 minutes on a horizontal rotator (Fisher Scientific Clinical Rotator) at 180rpm, circular path, and inverting the plates to empty. Plates were then incubated for 30 minutes with 1% (w/v) Bovine Serum Albumin (BSA) and emptied but not washed. Mouse sera were diluted 1/25 in PBS-T and TBW were used undiluted. A 0.2ml sample was added to each well and incubated for 2 hours. The plates were then washed as before and 0.2ml of conjugate diluted in PBS-T was added.

Conjugates, either HRPO or Alkaline phosphatase conjugated antimouse immunoglobulins were all titrated at less than 1/50. Following a 2 1/2 hour incubation period, the plates were again washed and 0.2ml of freshly prepared enzyme substrate added (5-aminosalicylic acid + 0.05% hydrogen peroxide for HRPO conjugates, p-nitrophenylphosphate in 10% diethanolamine buffer for Alkaline phosphatase conjugates)(Appendix 1). The plates were read after 1 hour by an 8 channel photometer (TitertekMultiskan, Flow Laboratories Inc., McLean, Va.) at 450nm for peroxidase conjugates or 405nm for alkaline phosphatase conjugates.

I. Double Antibody Indirect ELISA

Antigen

The antigen employed in this ELISA was a sonicate of *B. pertussis* strain 1494, prepared and titrated (to 5 ug/ml) as described previously for the indirect ELISA.

Samples

Individual serum samples were tested in duplicate. The samples were diluted 1/50 in PBS-T for IgG assays and 1/25 for IgA and IgM assays. Sera were absorbed with *Staphylococcus* Protein A before measuring IgM antibodies. TBW samples, pooled from the 5 mice per sample date, were used undiluted and tested in duplicate. Pooled positive sera or TBW maintained in aliquots at -20°C. were run as controls on all plates.

Anti-mouse Immunoglobulins

The rabbit anti-mouse immunoglobulins as listed for the indirect ELISA were used and were diluted in PBS-T (see Table 2 for dilutions).

Conjugate

The conjugate used in the double antibody indirect ELISA was commercially prepared Alkaline phosphatase anti-rabbit IgG (Goat) (Miles Laboratories, Elkhart, Indiana) diluted in PBS-T.

The optimal dilutions of conjugate and each anti-mouse immunoglobulin were determined by titration and are shown in Table 2.

Table 2

Optimum anti-mouse immunoglobulin and conjugate dilutions (presented as reciprocals of dilutions).

Antimouse Immunoglobulin	Antiserum Dilution	Conjugate dilution
Anti IgA	30,000	300
Anti IgM	5,000	800
Anti IgG1	1,000	450
Anti IgG2a	15,000	450
Anti IgG2b	120,000	1,200

Substrate

P-nitrophenylphosphate (PNPP) (Sigma 104 phosphatase substrate tablets) was diluted to 1mg/ml in 10% diethanolamine buffer (Appendix 1).

Double Antibody Indirect ELISA Protocol

See also summary Table 3

The following procedure is a modification of Granfor's(1979) method of measuring *Yersinia* antibodies by an ELISA. Microtiter plates (Dynatech polystyrene, flatbottom) were sensitized overnight with antigen, washed three times with PBS-T and incubated with 1% BSA (at 36°C) as previously described for the Indirect ELISA. Sera or TBW samples were added in 0.2ml amounts and incubated for 2 hours at 36°C. After washing the plates three times, 0.2ml of rabbit anti-mouse immunoglobulin was added to each well and the plates again incubated for 2 hours at 36°C. Alkaline phosphatase conjugated anti-rabbit IgG was added after a further 3 washes. Following another 2 hour, 36°C incubation and a last 3 washes, 0.2ml substrate was added. The enzyme reaction was read after 30 minutes incubation at 36°C on an 8 channel photometer (Titertek MultisKan).

Table 3

Double Antibody Indirect ELISA Protocol:

Sensitize plate with antigen, 4°C overnight.

↓ wash 3 times

Incubate for 30 minutes with 1% BSA at 36°C.

↓ empty

Add serum samples. Incubate for 2 hours at 36°C.

↓ wash 3 times

Add rabbit anti-mouse immunoglobulin. Incubate for 2 hours at 36°C.

↓ wash 3 times

Add alkaline phosphatase conjugated anti-rabbit IgG. Incubate for 2 hours at 36°C.

↓ wash 3 times

Add enzyme substrate. Incubate for 30 minutes at 36°C and read results.

J. Absorption of Antisera to Remove Crossreacting Antibodies

Two of the rabbit anti-mouse immunoglobulins (IgG1 and IgM) contained antibodies directly crossreactive with *B. pertussis* antigen. The crossreacting antibodies were eliminated by absorption with a suspension of formalin killed *B. pertussis* cells (strain 1494). The growth from approximately 18 CHBA plates was washed twice in FTA buffer and the cells were then resuspended in a minimal volume of FTA buffer with 0.05% formaldehyde. 1.0ml of the antiserum was added to the cell suspension and incubated at 37°C for 3 hours with occasional shaking. The absorbed antiserum was collected following centrifugation (4430g X 20 minutes).

K. Class Specificity of Anti-mouse Immunoglobulins

Class specificity of the anti-mouse immunoglobulins-Classes A, M, and G (subclasses G1, G2a, G2b) was determined by testing with purified mouse immunoglobulins: IgA (lot DG078), IgM (lot CJ169), IgG1 (lot BC023), IgG2a (lot CC049), and IgG2b (lot 231-58-2) (obtained from Bionetics Laboratory Products, Kensington, Maryland) by the ELISA. For example, anti-IgA was reacted with purified IgA, IgM, IgG1, IgG2a, and IgG2b to determine its specificity.

Tripling dilutions from 10ug/ml of the purified mouse immunoglobulins were made in coating buffer and sensitized in 0.2ml amounts to microtiter plates overnight at 4°C. Each of these dilutions were then reacted with 0.2ml of the five

anti-mouse immunoglobulins (at the optimal concentrations as determined for the ELISA) for 2 hours at 36°C. Alkaline phosphatase conjugate, (also added at the optimal concentrations as determined for the ELISA) was then added in 0.2ml amounts for 2 hours at 36°C. Finally, 0.2ml amounts of substrate were added, and the enzyme reaction read after 30 minutes incubation at 36°C. There were three 3 minute washes with PBS-T between each step as described in the ELISA protocols.

L. Absorption of Sera with Protein A

Protein A absorption of sera was carried out in an attempt to reduce nonspecific background in the IgM assays due to the ability of Protein A to remove IgG. Protein A was prepared and used according to the method employed by the Virology Department, Provincial Laboratory of Public Health, Edmonton (R.Devine, personal communication).

Protein A Preparation

A single colony of *Staphylococcus aureus*, Cowan 1 strain was inoculated into a 10ml amount of Trypticase Soya broth and incubated overnight at 37°C. The 10ml broth culture was added to one liter of modified CCY medium (Appendix 1) and placed on a magnetic stirrer at 37°C. A sterilized air flow was bubbled into the media allowing optimum aeration without excessive frothing. The escaping air was passed through water resulting in enough back pressure to alleviate frothing of the cultures. Bacteria

were harvested by centrifugation (1110g X 1 hour) and washed twice with PBS. The washed bacteria were made up to a 10% suspension in PBS and fixed in 0.5% formaldehyde for 3 hours at 4°C, followed by two more washes in PBS. The fixed 10% suspension was then heat fixed in a double boiler at 80°C for 30 minutes. After heat treatment, the bacterial suspension was washed once more, made up to a 10% suspension, and stored at 4°C.

Absorption of Sera

0.5ml of the 10% staph Protein A suspension was centrifuged 20 minutes at 4000rpm (clinical centrifuge) and the supernatant removed. An equal volume (0.5ml) of a 1/25 serum dilution was added to the pelleted cells and incubated for 20 minutes at room temperature. The suspension was centrifuged as before and the supernatant removed to be used in the ELISA.

M. Standardization of Results

Due to day to day variations in ELISA results, the method of Mansheim and Kasper (1979) was used to standardize results. A positive control serum was run with each day's tests. The results of samples tested on different days were standardized to Day 1 by applying the following formula: (control serum Absorbance on day 1)/(control serum Absorbance on day 'x') times (test serum Absorbance on day 'x') = test serum Absorbance on day 1.

III. Results

A. Experiment 1: Infection in mice following intranasal instillation of *B. pertussis*

Detection of B.pertussis by culture and immunofluorescence

Intranasal instillation of 2.5 X 10^5 CFU of *B. pertussis* into 6 week old mice initiated a non lethal respiratory tract infection although no obvious signs of disease in the mice were noted. A high incidence of recovery of organisms by culture from TBW, trachea, or lung was found during the first 3 weeks following inoculation but thereafter only occasional mice yielded positive results (Table 4). The last positive culture was obtained on day 44 after inoculation. Culture of TBW (24 positive), or trachea (26 positive) yielded slightly better results than culture of lung tissue (21 positive) (Table 5). Results of TBW plated directly or cultured by the enrichment method of Reagan and Lowe (1977) were the same.

Although the majority of cultures taken after 3 weeks of infection were negative, organisms were detectable by direct immunofluorescence for a much longer time (Table 4). Almost all TBW's collected until the 39th day of infection were FA positive, and the incidence of positive FA results remained high until at least day 129. In the final group of mice sacrificed at day 177, no *B. pertussis* was detected by immunofluorescence.

Only scant *B. pertussis* was present in all slides identified as FA positive and for the most part these were typical, highly fluorescent coccobacilli. Larger, atypical organisms, also highly fluorescent were very occasionally noted but the slides containing these organisms also contained typical *B. pertussis*.

All samples collected from control mice housed in a separate room were negative for *B. pertussis* by culture and immunofluorescence.

Table 4

Results of culture and direct immunofluorescence on samples from groups of five mice, sacrificed at the indicated times following inoculation with *B. pertussis*. (Experiment 1)

Day after Inoculation	Mouse # 1	2	3	4	5	
4	+	+	+	+	+	C
	+	+	+	+	+	FA
8	+	-	+	+	-	C
	+	+	+	+	+	FA
11	-	+	-	+	+	C
	+	+	+	+	+	FA
16	+	+	+	-	+	C
	+	+	+	+	+	FA
18	+	+	+	+	+	C
	+	-	+	+	+	FA
21	+	-	-	+	+	C
	-	+	+	+	+	FA
24	+	-	-	-	-	C
	+	+	+	+	+	FA
28	-	ns	-	-	-	C
	+		+	+	+	FA
32	-	-	-	-	+	C
	+	+	+	+	+	FA
36	+	-	-	-	+	C
	+	+	+	+	+	FA
39	-	-	-	-	-	C
	+	+	+	+	+	FA
44	-	-	-	+	-	C
	+	-	+	+	-	FA
	All subsequent cultures negative					
49	-	+	+	-	-	FA
53	+	+	-	-	+	FA
58	-	+	+	-	+	FA
63	-	+	+	-	+	FA
70	-	-	+	-	-	FA
77	-	+	-	-	+	FA
84	+	-	+	-	+	FA
92	-	+	+	+	+	FA
102	-	-	+	+	-	FA
114	+	-	+	-	-	FA
129	+	+	-	-	-	FA
177	-	-	-	-	-	FA

C = Culture
FA = Immunofluorescence
ns = no sample

Table 5

Recovery of B. *pertussis* from tracheobronchial wash, trachea and lung of inoculated mice.

Day after Inoculation	Source of Sample	Mouse # 1	2	3	4	5
4	TBW	+	+	+	+	+
	Trac.	+	+	+	+	+
	Lung	+	+	+	+	+
8	TBW	+	-	+	+	-
	Trac.	+	-	-	+	-
	Lung	+	-	-	-	-
11	TBW	-	+	-	+	+
	Trac.	-	+	-	+	+
	Lung	-	+	-	+	+
16	TBW	+	+	+	-	+
	Trac.	+	+	+	-	+
	Lung	+	+	+	-	+
18	TBW	+	-	+	-	+
	Trac.	+	+	+	+	+
	Lung	+	+	-	+	+
21	TBW	+	-	-	+	+
	Trac.	+	-	-	+	+
	Lung	-	-	-	+	+
24	TBW	+	-	-	-	-
	Trac.	+	-	-	-	-
	Lung	+	-	-	-	-
28	TBW	-		-	-	-
	Trac.	-	ns	-	-	-
	Lung	-		-	-	-
32	TBW	-	-	-	-	-
	Trac.	-	-	-	-	+
	Lung	-	-	-	-	-
36	TBW	+	-	-	-	-
	Trac.	+	-	-	-	+
	Lung	-	-	-	-	+
39	TBW	-	-	-	-	-
	Trac.	-	-	-	-	-
	Lung	-	-	-	-	-
44	TBW	-	-	-	+	-
	Trac.	-	-	-	-	-
	Lung	-	-	-	-	-

TBW = Tracheobronchial Wash
Trac. = Trachea
ns = no sample

Immunological Response to pertussis infection in mice

a. Optimal conditions for ELISA technique:

1. Preparation of conjugates for ELISA

Initially, several attempts were made to prepare conjugates for each of the major immunoglobulin classes of mouse antibody by coupling enzymes with commercially prepared anti-mouse immunoglobulins (A, M, G1, G2a, and G2b). Both Horse Radish Peroxidase (HRPO) and Alkaline Phosphatase were used as enzyme markers. Gel filtration was used to separate free enzyme and immunoglobulin from the conjugate. Since only one peak corresponding to both protein and enzyme was observed (Figures 1 and 2), for all conjugates, it can be assumed that there was 100% conjugation with both HRPO and Alkaline phosphatase. However, upon titration, the activity of all the conjugates was found to be so low that they proved unsuitable for use in the ELISA test.

Figure 1: Elution (with 2 ml fractions) of Horse Radish Peroxidase conjugated anti-mouse IgA from a Sephadex G200 column equilibrated with PBS pH 7.2.

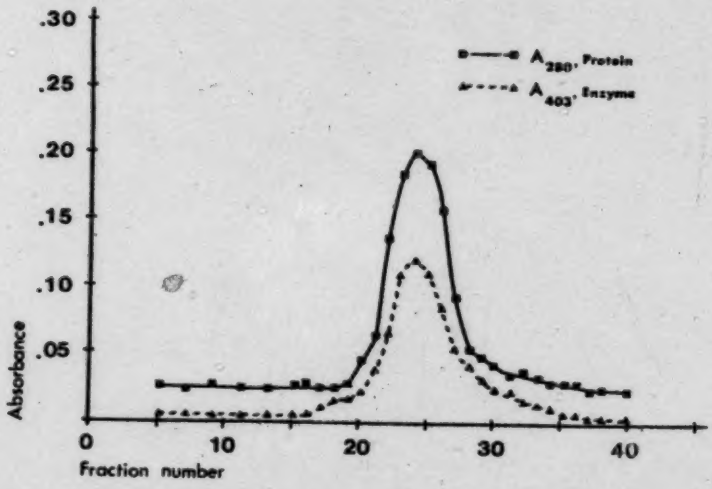
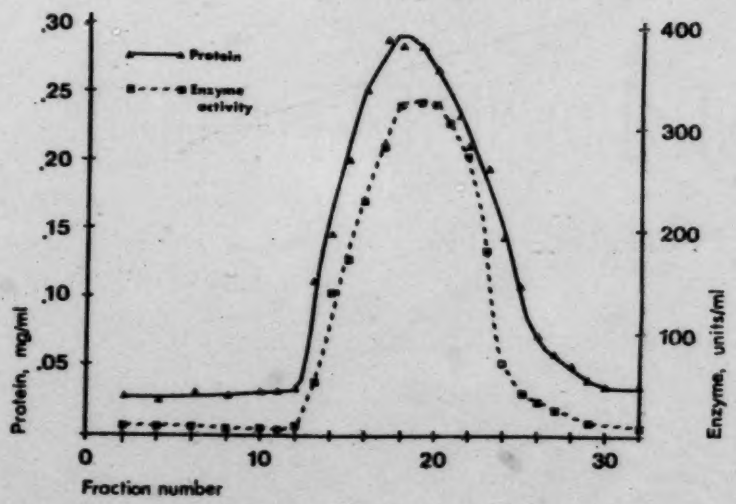


Figure 2: Elution (with 2.5ml fractions) of Alkaline phosphatase conjugated anti-mouse IgA from a Sephacryl S300 column equilibrated with TRIS pH 8.0.



2. Double Antibody Indirect ELISA

A double antibody indirect ELISA was therefore developed which used unconjugated anti-mouse immunoglobulins (rabbit). The conjugate was commercial Alkaline phosphatase anti-rabbit IgG. This system proved to be very sensitive and allowed high dilutions of both antisera and conjugate (See Table 2 in Materials and Methods).

3. Class Specificity of Anti-mouse Immunoglobulins

The specificity of the anti-mouse immunoglobulins was tested by reacting them on ELISA plates to which chromatographically purified mouse immunoglobulins had been added at varying dilutions. The results of these experiments (Figures 3 to 7) revealed that anti-IgG1, IgG2b and IgM were specific for their homologous immunoglobulins. However, anti-IgA and anti-IgG2a showed some degree of cross reactivity with other immunoglobulin classes: anti-IgA with both, IgM at a level of about 1/10 the activity with IgA, and anti-IgG1 at insignificant levels. Anti-IgG2a crossreacted with IgG2b at levels ranging from 1/2 to 1/10 that of IgG2a depending on the concentration of the immunoglobulin present.

Figures 3-7 represent the reactivity of anti-mouse immunoglobulins on wells that had been coated with purified mouse immunoglobulin. The concentration of immunoglobulin used to coat the wells is indicated on the abscissa.

Figure 3

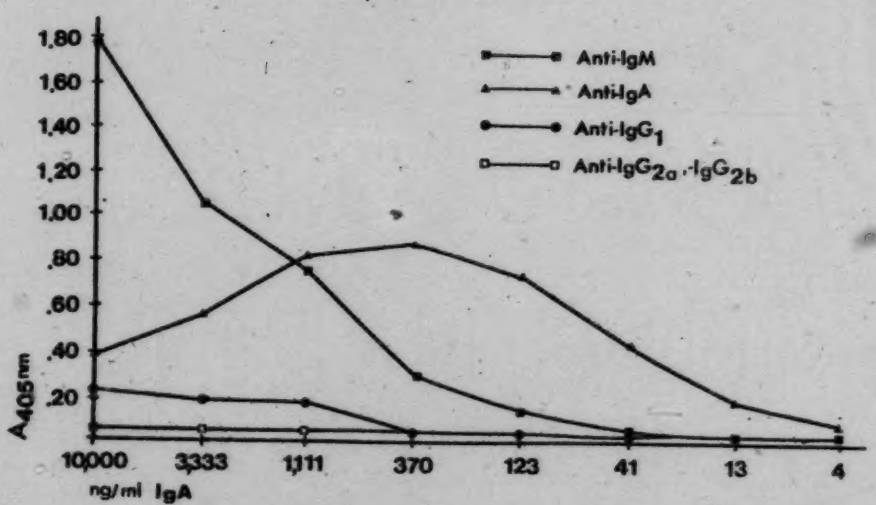


Figure 4

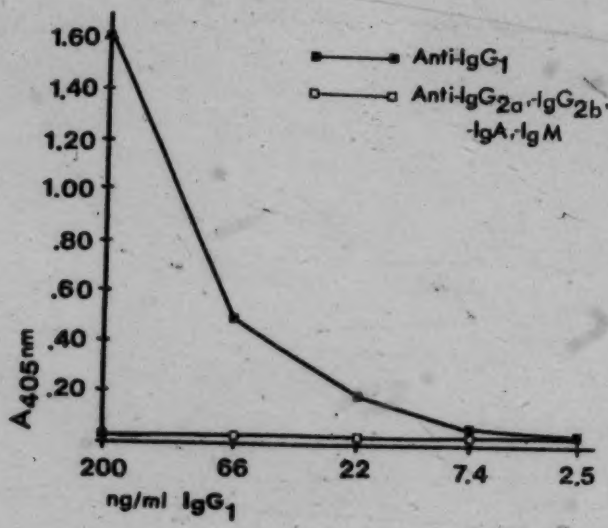


Figure 5

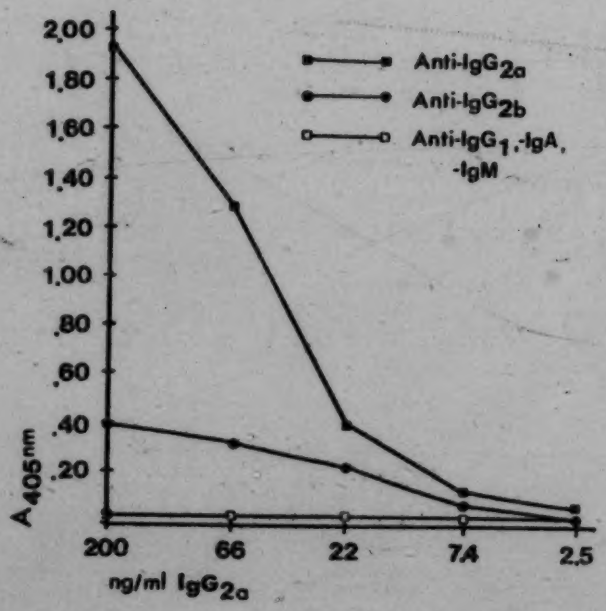


Figure 6

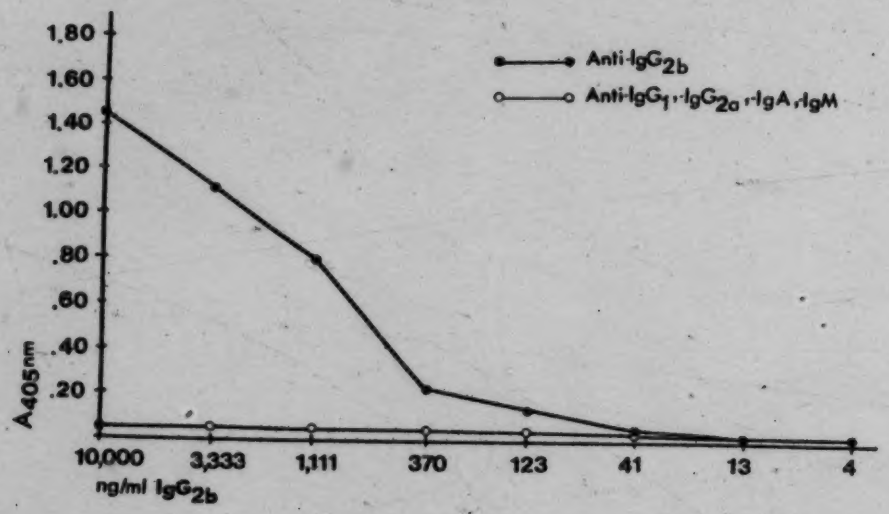
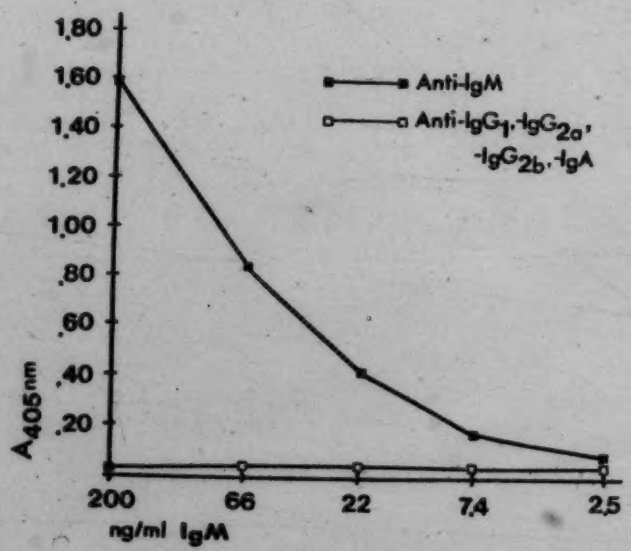


Figure 7



Serum Immunoglobulin Responses

The pertussis specific antibody responses of five serum immunoglobulin classes as determined by the Double Antibody Indirect ELISA are shown in Figures 8 to 12. For each sampling date, a single dilution of serum from each of the 5 mice was run separately by the ELISA and the results (absorbance value at 405nm) averaged. The data plotted for each group of mice represents the mean absorbance value plus or minus one standard deviation. Control mice showed no response for IgG2a and IgG2b. Absorbance values for IgA, IgM and IgG1 in control mice are shown on the graphs where the data represents the range between the two control values.

General trends in the immune response were observed even though there were often rather wide variations between the readings of each immunoglobulin class per group (as demonstrated by the standard deviation ranges). Pertussis specific antibodies reached detectable levels by about day 16 to 18 after infection and were still present at day 177 when the last group of mice were sacrificed. IgG1, IgG2a, and IgG2b increased until the 7th to 8th week and then gradually declined. Although IgM appeared at the same time as IgG, peak levels were reached earlier from about day 20 to 50 before declining. Results obtained with serum IgA were more difficult to interpret. There was a rather wide variation in results from different mice but the overall pattern indicated a continued increase in IgA levels with no obvious peak being reached by the termination date of the

experiment. Complicating matters further with the IgA results was that the IgA levels in control mice also appeared to increase, although not to the same extent as the samples from the infected mice.

Figures 8-12 represent the pertussis specific antibody responses in serum from mice intranasally inoculated with *B. pertussis* where the disease was followed for 177 days and serum antibody levels measured by ELISA. The results from infected mice are shown by a solid vertical line representing ELISA absorbance values in the form of the mean plus or minus one standard deviation from five mice. Control mice results are shown by a broken line which represents the range between two mouse responses.

Figure 8

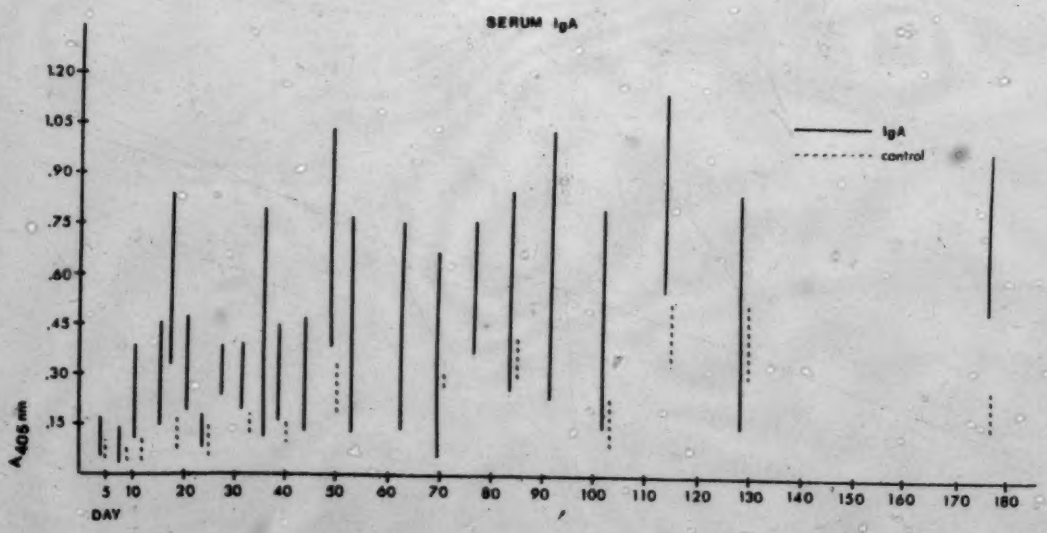


Figure 9

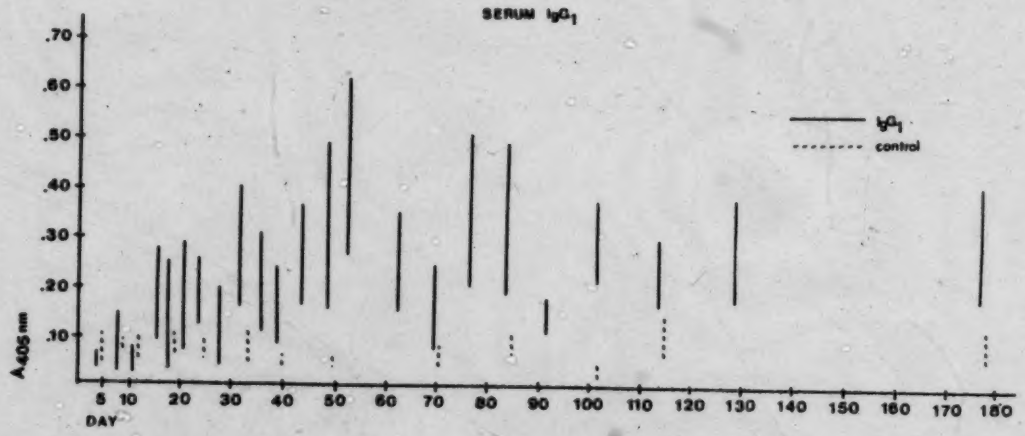


Figure 10

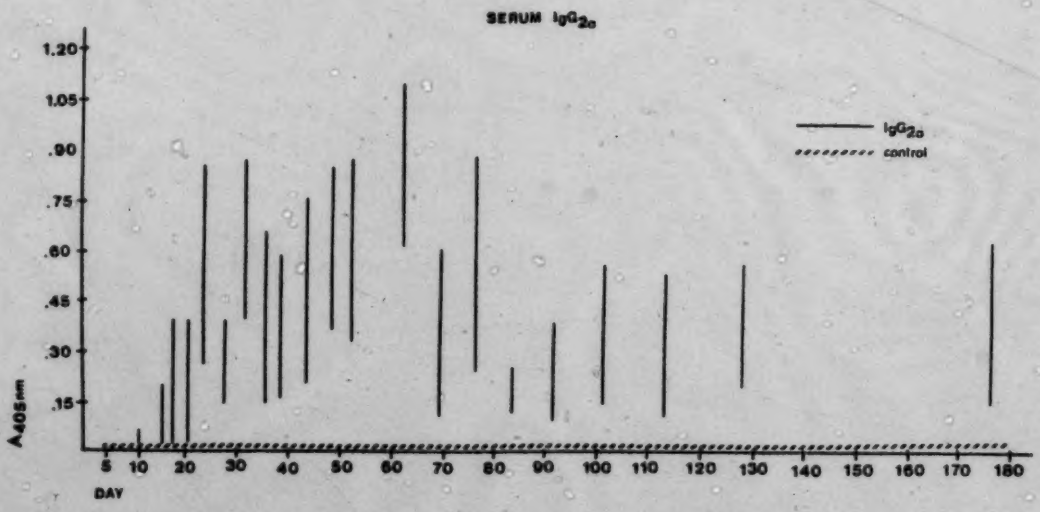


Figure 11

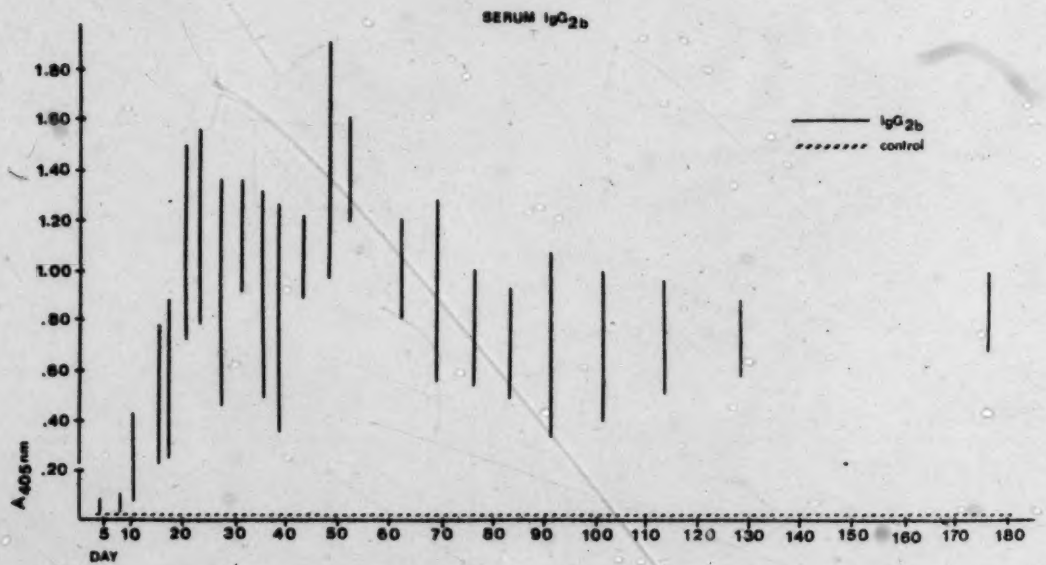
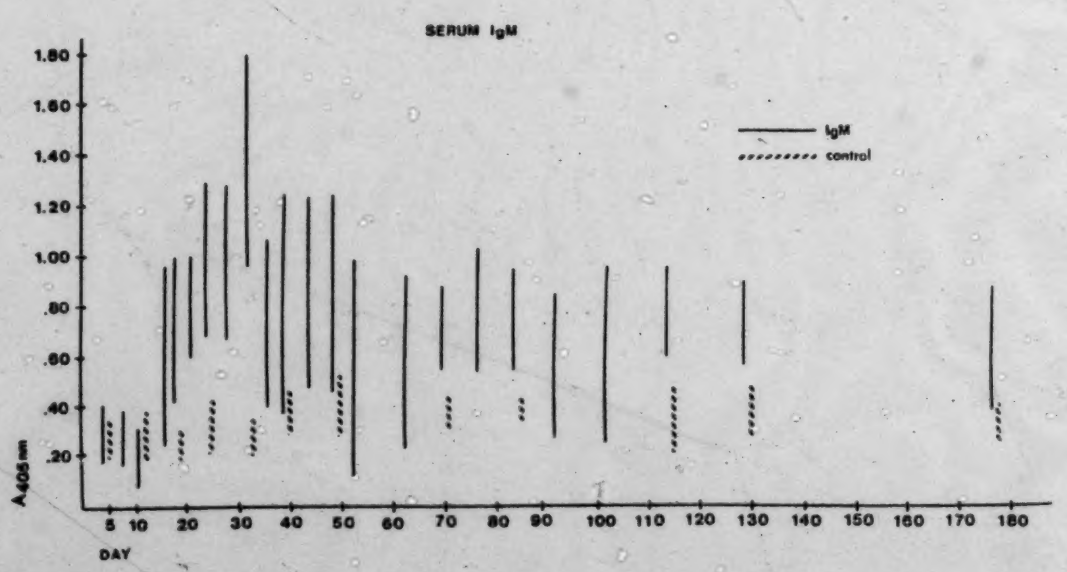


Figure 12



TBW Immunoglobulin Responses

Pooled TBW's from Experiment 1 were tested by ELISA for the presence of pertussis antibodies. Results are shown in Figures 13 to 16 where each data point represents a pool of TBW's from five mice. Values for control mice were all zero except for IgG1 in which the results were minimal from the pooling of 2 TBW's.

Pertussis specific IgA and IgG (subclasses G1, G2a, and G2b) were detected but there was no detectable IgM response. IgA was present by day 18, peaked by about day 30, and began to decline in the 7th to 8th week of disease although it was still present at day 177. The three subclasses of IgG were present in TBW but the responses were not as strong as that of IgA. Of the three, IgG1 gave the strongest response and began to increase after day 20. IgG2a was detectable slightly earlier than IgG1 and IgG2b. No strong peak period was observed with any of the IgG's and there was a decline after about day 80. In this decline period, several samples reached negative values, unlike TBW-IgA and serum IgG responses.

Figures 13-16 represent the pertussis specific antibody responses in TBW from intranasally inoculated mice with *B. pertussis* where the disease was followed for 177 days and TBW antibody responses were determined by ELISA. The results from infected mice are shown as a solid line where each point represents the ELISA absorbance value from a pool of

five mouse TBW's. Control mice results are indicated by a broken line and each point represents the ELISA absorbance value from a pool of 2 TBW's.

Figure 13

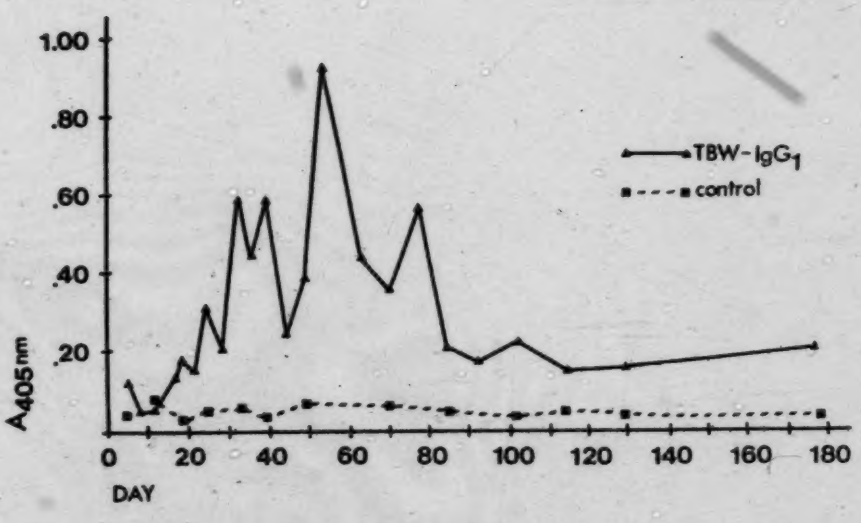


Figure 14

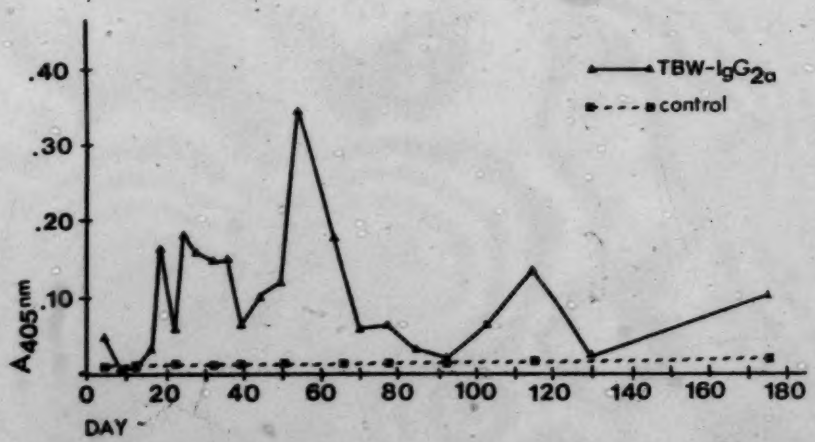


Figure 15

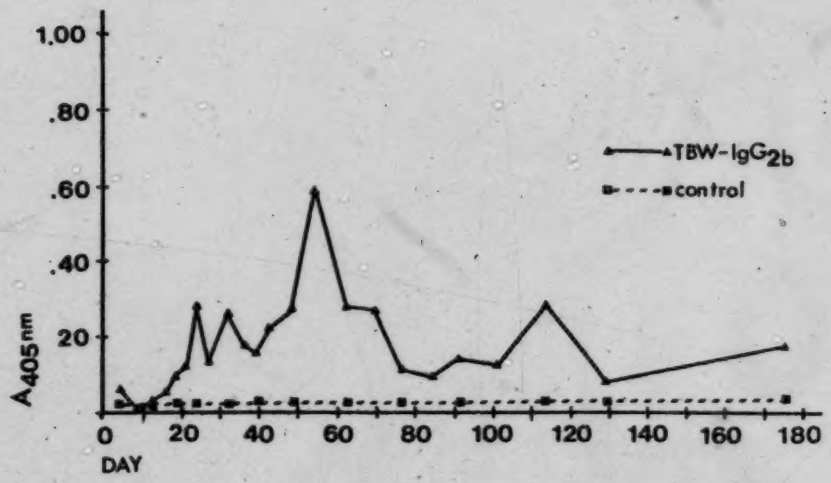
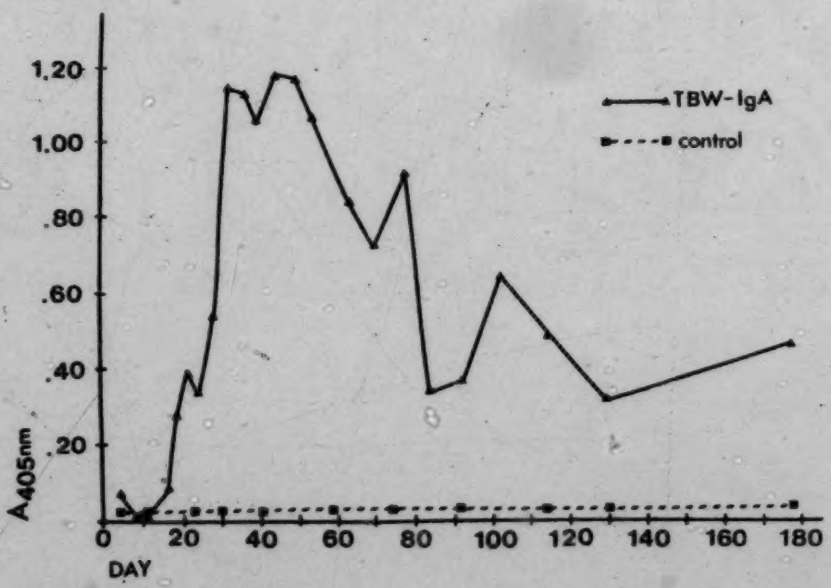


Figure 16



B. Experiment 2

Results from Experiment 2, in which uninoculated mice were exposed to infected mice are shown in Table 6. Although at no time were exposed mice culture positive for *B. pertussis*, many animals showed antibody responses and often *B. pertussis* was seen by FA. Response in a single immunoglobulin class may not be indicative of infection but numerous mice showed multiple responses and therefore were considered as having been infected. Responses with minimum ELISA absorbances of 1.0 for IgM, 0.4 for IgG1, 0.5 for IgG2a, 1.0 for IgG2b and 0.4 for IgA were considered as positive. Even after exposing uninoculated mice to mice that had been infected 9 weeks previously, the exposed mice still developed antibody responses.

Table 6

Results from uninoculated mice that had been exposed to infected mice at the times indicated after infection. Groups of 4 or 6 mice were sacrificed at 10, 20, or 30 day intervals following exposure. TBW samples were cultured, examined by direct immunofluorescence for *B.pertussis*, and tested for pertussis specific IgA. Serum samples were tested by ELISA for pertussis specific IgG1, IgG2a, IgG2b, and IgM. Only positive reactions are recorded in the table.

Day of exposure to infected mice	Day of sampling after exposure																	
		10						20						30					
	#	1	2	3	4			1	2	3	4			1	2	3	4		
7	FA	+		+				+		+	+					+			
	IgG1							+		+				+					
	IgG2a		+					+						+					
	IgG2b							+		+				+					
	IgM									+				+					
	IgA																		
14	FA	+		+											+	+	+		
	IgG1							+											
	IgG2a				+									+					
	IgG2b	+								+				+					
	IgM							+											
	IgA														+				
21	FA	+			+				+						+		+		
	IgG1																		
	IgG2a														+				
	IgG2b									+									
	IgM	+																	
	IgA								+			+							
	#	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
28	FA																	+	
	IgG1																		
	IgG2a	+																	
	IgG2b	+																	
	IgM	+																	
	IgA														+				
35	FA							+		+		+	+	+					
	IgG1																+		
	IgG2a																+		
	IgG2b	+																	
	IgM	+							+										
	IgA								+								+	+	
42	FA								+		+	+							
	IgG1																		
	IgG2a																		
	IgG2b																	+	
	IgM	+			+		+	+	+		+					+	+		+
	IgA													+		+	+		
49	FA	+		+	+		+									+			+
	IgG1							+							+				
	IgG2a							+						+			+		
	IgG2b													+			+		
	IgM			+		+		+						+	+	+	+	+	+
	IgA													+	+	+		+	
56	FA						+								+				
	IgG1													+					
	IgG2a										+								
	IgG2b																	+	
	IgM	+			+		+		+	+			+	+	+			+	
	IgA								+										
63	FA																		
	IgG1																		
	IgG2a						+							+	+				
	IgG2b																		
	IgM	+		+		+	+	+			+	+							
	IgA													+					

IV. Discussion

B. pertussis infection of the mouse respiratory tract was found to follow the course previously described for this model (Geller and Pittman, 1973)and resembled human pertussis in duration of infection, although the mice displayed no obvious symptoms of infection. Following initial infection of mice there was a high rate of recovery of the organisms by culture for approximately 21 days, followed by a rapid decline, although positive cultures were obtained from occasional mice until day 44. Even though organisms detectable by the culture methods employed in this study disappeared early in the disease, organisms detectable by direct immunofluorescence persisted for a much longer period. Most TBW's were positive by immunofluorescence up to about the 40th day after infection. Following this, there was a slight decline in the number of specimens exhibiting immunofluorescent organisms; however, organisms were still seen in 2 mice at the 129th day of infection.

False positives are reported to range from 6.7 to 40% of FA positive results (Broome et al, 1978) and the possibility therefore exists that some of the FA positive results recorded in this study were in fact false positives. However, the high incidence of false positives can often be explained in part by reader variability and inexperience. In this study the effects of both of these variables has been minimized as all observations were made by a single, experienced observer. In addition, the mouse TBW's contained

very little interfering background fluorescence so that fluorescing *B. pertussis* cells, if present, could be easily discerned.

In human pertussis, culture is more likely to be positive in the early stages of disease, but even at this time, it is not always successful. Less than 50% of untreated patients will have positive cultures after the 3rd week of illness and 20% or less after the 5th week (Linnemann, 1978). The difficulty in culturing organisms may be explained in part by lengthy handling and processing times of some specimens and in other cases by infections in which agents other than *B. pertussis* cause the pertussis syndrome. This study confirmed the difficulty of culturing *B. pertussis* even in the early stages of disease. Specimens of TBW, lung, and trachea from 2 out of 5 mice on the 8th and 11th days after infection failed to give positive cultures, although organisms were seen by direct immunofluorescence.

Several steps were taken to obtain the highest rate of *B. pertussis* recovery from infected mice. TBW's were inoculated directly onto fresh CHBA plates, even though it had been shown that the strain of *B. pertussis* used for initial inoculations of the mice was stable for at least 90 minutes in saline, the solution used to wash the respiratory tract. TBW's were also cultured by an enrichment method (Regan and Lowe, 1977) but this did not prove to be any more sensitive than direct plating of TBW's. In addition to

TBW's, portions of the trachea and lung were cultured for *B. pertussis*.

The detection of the appearance or increase in antibody titer against a microorganism is often used to confirm the suspected clinical diagnosis of an infectious disease. Moreover, when attempts to culture the organism have failed, determination of antibodies against the particular microorganism may be the only means of providing laboratory confirmation of the disease. Although antibodies have been detected in pertussis infection by a variety of techniques, the results of such serological tests may be unreliable for diagnosis in individual clinical cases. The presence of antibodies arising from immunization complicates the use of serum antibody levels for diagnosis.

In this study, an enzyme linked immunosorbent assay was used to follow pertussis antibody responses to investigate their importance in relation to diagnosis as well as their role in immunity. The introduction of the ELISA test for the determination of antibodies has afforded many new diagnostic potentials. ELISA has proven to be well suited for sensitive and accurate detection of the antibody response to many infectious diseases (Voller et al, 1976). A further advantage is that all immunoglobulin classes can be detected with equal efficiency so that the method allows class specific antibody determination if desired.

The sensitivity of enzyme immunoassays depends to a large extent on the preparation of enzyme-antibody

conjugates possessing high enzymatic and immunologic activity. Two types of coupling reactions have been described: one and two step reactions. In one-step procedures, the enzyme, the cross linking agent, and the immunoglobulin are all mixed together and allowed to react. In this procedure, the reaction is difficult to control and conjugates are heterogeneous. In two-step procedures, the enzyme is first treated with the cross linking agent and then the immunoglobulin is added. Theoretically, in the two step procedure, the reaction is easier to control than the one step procedure, and more homogeneous conjugates are achieved.

Sodium periodate is the agent of choice for coupling peroxidase to antibodies. Conjugate preparations for this study were made using this one step procedure. However, initial attempts resulted in conjugates of very low activity. Alterations to the original sodium periodate method were subsequently performed in an attempt to increase the activity of HRPO-conjugates. The sodium borohydride step (stabilizing agent) was omitted since it has been observed that while the enzymatic activity remains high after sodium borohydride treatment, the antibody activity of the conjugate is reduced 50 to 60% (Saunders, 1979). Sodium borohydride was also deemed unnecessary since the conjugates would be used within a relatively short period of time.

Several authors used increased concentrations of immunoglobulins over the traditional 1:1 ratio of enzyme to

immunoglobulin when coupling (eg. Saunders, 1979; Mathieson et al, 1978) with the intent of obtaining a larger percentage of one enzyme molecule to one immunoglobulin molecule and thereby improving conjugate activity. However, with this and the sodium borohydride modifications to the coupling procedure, HRPO conjugates could not be produced with sufficient activity to warrant use. Avrameas et al (1978) stated that the immunological and enzymatic activity of peroxidase conjugates as compared with precoupling values was 17% and 30% respectively. However, conjugates prepared with a two-step glutaraldehyde procedure exhibited 50% and 50-75% of the initial immunological and enzymatic activity, respectively. It was suggested (R. Haufe, personnal communication) that Alkaline phosphatase when coupled by glutaraldehyde gave better conjugates than those with Horse Radish Peroxidase. Alkaline phosphatase conjugates produced in this manner were found to be superior to HRPO conjugates but still of insufficient activity for use in an indirect ELISA.

The conjugate is the most expensive component of the ELISA technique and it is therefore not feasible to use conjugates of low activity. The activity of all conjugates produced for this study were very low (optimally diluted at less than 1/50) and therefore, another form of ELISA, the double antibody indirect ELISA was developed. This system employed an extra antibody step, which served to amplify the reaction and also, circumvented the need to conjugate each

individual anti-mouse immunoglobulin. An added advantage was that it made possible the use of a commercially available conjugate.

The protocol for the double antibody indirect ELISA was a modification of Granfor's (1979) method. This ELISA proved to be a highly sensitive and specific test. Sensitivity of the test is indicated by the high degree of dilution for both immunoglobulin and conjugate. Evidence for sensitivity and specificity in the test also comes from the testing of anti-mouse immunoglobulins with purified mouse immunoglobulins (Figures 3 to 7). The data indicated that the test was sensitive at less than 100ng of immunoglobulin per ml. Anti-IgM, -IgG1, and -IgG2b were specific for their homologous immunoglobulin. However, anti-IgA was cross reactive with both IgM and to a lesser, and probably insignificant extent, with IgG1. The cross reaction with IgM is unlikely to be significant when measuring IgA levels in TBW as no IgM was detected here. This cross reaction was a possible source of error in the measurement of serum IgA levels. However, if an ELISA absorbance value of 0.3 to 0.4 were considered as positive, the crossreactivity with IgM was only about 1/10 the activity of IgA.

Anti-IgG2a was significantly cross reactive with IgG2b. However, since this cross reaction was confined to the IgG2 subclass, it's importance is diminished.

If temperatures of 36°C were used in the ELISA, incubations could be completed within 2 hours for most

steps. Initially, high background values were present in the test; probably due to nonspecific binding of either antibody or conjugate (Saunders, 1979) although the high incubation temperatures may also have had a part in this. During incubation times, all proteins will adhere to available sites on the solid phase and eventually, all available sites are taken up by one molecular type or another. One method of lowering non-specific binding is to add Bovine Serum Albumin (BSA) to the antibody diluent to give a concentration of BSA much greater than that of immunoglobulin. Consequently, more BSA molecules than immunoglobulin molecules bind to available sites on the solid phase. Instead of including BSA in the diluent, an incubation with 1% BSA after sensitizing plates with antigen was sufficient to prevent non-specific binding and thus lower the background values.

General trends in the immune response to pertussis infection in mice were observed although there were often rather wide variations between the absorbance values obtained within a particular sampling date and also between sampling dates. This was most likely due to the degree of infection attained by each mouse. The utmost care was taken when performing intranasal inoculations to ensure that all mice received the same amount of inoculum. However, the extent of infection could not be determined and individual variations between mice in their response to pertussis probably accounts for a large amount of the disparity between results.

Increased serum levels of pertussis antibody of the five immunoglobulin classes tested (IgA, IgM, IgG1, IgG2a, IgG2b) were observed. The antibodies began to appear early in the third week of disease and persisted until at least the 25th week. IgG levels appeared to peak 50 to 60 days after infection whereas IgM peaked somewhat earlier in the 20 to 50 day range.

Initial attempts to measure IgM levels were unsuccessful as absorbance values of sera from control mice were not significantly different from those of infected mice. To determine if removal of IgG would improve the detection of IgM, the mouse sera were absorbed with Protein A. The absorbed sera proved to be more satisfactory for the pertussis IgM assay although relatively high absorbance values were still obtained in the sera from control mice.

The serum IgA response was difficult to analyze since values in control mice appeared to increase along with that of the infected mice. Within the time period observed, it could not be determined if a maximal level of serum IgA was attained.

Geller and Pittman (1973) measured the immunoglobulin response to pertussis infection in mice and also observed an interesting response with serum IgA. They noted a large rise in serum IgA levels in relation to the other immunglobulin classes 30 days after intranasal infection. However, this increase was determined from a small sample and antibody specificity of the IgA was not ascertained. Furthermore, the

study was completed in the relatively short time period of 50 days.

The role of serum antibody in resistance to infection or re-infection remains unresolved. Increased agglutinating antibody levels correlate to some extent with increased immunity although pertussis can develop in the presence of antibody (Linnemann, 1978). That serum antibody may play a role in resistance to infection is suggested by the observation that parenteral inoculation with killed *B. pertussis* organisms gives rise to a resistance to infection. This immunity, which may persist for several years implies, the presence of a persistent immunological memory and suggests that IgG plays an important role in prolonged resistance to infection (Olsen, 1975).

Pertussis is a superficial infection and one of the difficulties in evaluation of the importance of the immune response has been the lack of consistent findings on the relationship between antibody levels and events in the respiratory tract.

Pertussis specific IgA and three IgG subclasses (G1, G2a, G2b) were present in washings of the mouse respiratory tract after intranasal inoculation with *B. pertussis*, but no IgM was detected. IgA appeared on about day 18 after infection, reached a strong peak by about the 4th week, and was still present 25 weeks later (end of this study).

IgG appeared at approximately the same time as IgA although at much lower levels. Maximal levels were attained

between approximately the 30th and the 70th day of infection. The apparent peak on day 58 is difficult to explain.

IgA is the predominant class of antibody in secretions bathing the mucus membranes. The mechanism of IgA action has not been fully explained. IgA is known to neutralize viral activity, but less is known about it's antibacterial functions. Secretory IgA is not generally considered to be bactericidal, to mediate complement dependent lysis (at least by the classical pathway, although its action by the alternate pathway remains a possibility), or to bind to macrophages or enhance phagocytosis (Tomasi, 1970). However, the recognized ability of secretory IgA to bind specifically to bacterial cells and affect their aggregation could influence their adherence (Williams and Gibbons, 1972). Hence, the major mechanism of immunity mediated by secretory IgA is likely to be the prevention of attachment of bacteria to the ciliated epithelium.

IgG in secretions is present at highest concentrations in the lower respiratory tract (Reynolds et al, 1970). Some of it is probably present from increased vascular permeability (serum leakage) as a result of inflammation. However, *Mycoplasma pulmonis* IgG1 and IgG2 containing cells have been demonstrated in the mouse respiratory tract indicating the possibility of some local production (Taylor and Howard, 1980).

The IgG in secretions, although only in low levels,

probably plays more of a bactericidal role than IgA. IgG2 can promote phagocytosis or complement mediated lysis. However, it is not clear how IgG1 might mediate resistance since it is not opsonic and it's ability to fix complement is uncertain (Taylor and Howard, 1980).

Several postulates can be drawn from studying the antibody responses to pertussis. The disappearance by about 20 days after infection of the majority of organisms in culture correlates well with the appearance of peak levels of secretory IgA. The specific role of IgA would probably be to prevent attachment of *B. pertussis*, with the antibody being directed against the attachment appendages-possibly the hemagglutinin (Sato et al, 1978).

Secretory IgA arises only in response to actual infection by the organism and not parenteral vaccination. The detection of pertussis IgA from the respiratory tract may be a useful procedure for the retrospective diagnosis of pertussis and also aid in the differentiation between true pertussis and the pertussis syndrome associated with other agents (Goodman et al, 1981). The early appearance and persistence at relatively high levels for several months of pertussis IgA could be an invaluable diagnostic tool, if combined with the clinical picture.

Direct immunofluorescent examination of TBW's from mice after infection with *B. pertussis* indicated that the organisms persisted for several months after cultures became negative. An experiment was therefore initiated to determine

if these immunofluorescent organisms were capable of transmitting infection. Uninoculated mice were exposed to infected mice at weekly intervals for up to 9 weeks. At 10, 20, and 30 day intervals after exposure, groups of exposed mice were sacrificed. Samples of TBW were cultured, examined by direct immunofluorescence for *B. pertussis* and tested for pertussis specific IgA by ELISA. Serum samples were tested by ELISA for the presence of pertussis IgG1, IgG2a, IgG2b, and IgM. Positive ELISA absorbance values were chosen as being approximately one half of the peak response level attained for that antibody during the immune response. Serum IgA and TBW-IgG were not included in the study due to the difficulty of interpreting these responses in the immune response experiment.

At no time was *B. pertussis* cultured from exposed mice. However, direct immunofluorescence of TBW indicated the presence of *B. pertussis* in many of the exposed mice. Response in a single immunoglobulin class or by FA alone may not have been indicative of infection but many animals showed multiple responses and therefore good evidence of infection.

The work of Gray and Cheers (1967) indicated that *B. pertussis* could survive intracellularly in alveolar macrophages in a complaisant state for weeks after infection. Dolby et al (1961) also noted that in many cases, mouse lungs harbored small numbers of viable *B. pertussis* for months . Although it has been shown that *B. pertussis* is

capable of surviving for long periods of time after infection, possibly intracellularly, it has not been determined if these organisms constitute a potential infective source.

In this study it was shown that *B. pertussis* could not be cultured from exposed uninoculated mice, although direct immunofluorescence and antibody results indicated that the organisms were transmissible for at least 9 weeks. Since the organisms could not be cultured from the exposed mice, it is possible that the organisms were present in an altered form in both infected and exposed mice. Such an altered form of the organism, if present in the human, could constitute a potential source of infection leading to sporadic outbreaks of the disease. The fact that the organism may be able to exist in the presence of peak antibody levels indicates that immunization may do no more than render the organism a commensal rather than a pathogen in an immunized host.

The inability to culture the majority of organisms past the third week of infection may be a result of one or both of the following events: 1. Organisms move to an intracellular environment 2. Organisms may assume an altered state and no longer be culturable by standard bacteriological methods.

This study has brought to light several interesting features of the mouse pertussis model and the results suggest the possibility of the existence of a carrier state. While further study is necessary, the findings open up

intriguing possibilities for the maintenance of *B. pertussis* in human populations.

Further study in this area could involve an expansion of the transmission experiment with larger numbers of mice and more frequent sampling procedures. In addition to the culture, immunofluorescent and antibody studies employed for this experiment, it might be useful to attempt culturing the organisms from homogenated lung specimens to determine if organisms are intracellular and viable. In order to determine the extent of *B. pertussis* infectivity a more precise investigation of the time period for transmission would be necessary, as in the present study, uninoculated mice were exposed for a minimum of 10 days of continuous intimate contact.

V. Bibliography

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VI. Appendix 1

A. Buffers

Carbonate-Bicarbonate Buffer;pH 9.6 (Coating Buffer)

(Voller, 1976)

Sodium carbonate (Na_2CO_3)	1.59g
Sodium bicarbonate ($NaHCO_3$)	2.93g
Sodium azide (NaN_3)	0.20g
Distilled water	1000ml

Phosphate Buffered Saline;pH 7.4 (PBS)

(Voller, 1976)

Sodium chloride (NaCl)	8.00g
Potassium phosphate monobasic (KH_2PO_4)	0.20g
Sodium phosphate dibasic (Na_2HPO_4)	1.15g
Potassium chloride (KCl)	0.20g
Distilled water	1000ml

Phosphate Buffered Saline-Tween;pH 7.4 (PBS-T)

(Voller, 1976)

Add 0.5% Tween 20 to PBS

TRIS; pH 8.0

0.05M Tris-Hydroxymethylaminomethane in distilled water.

FTA Hemagglutination Buffer;pH 7.2 (FTA Buffer)

(BBL, Cockeysville, Md.)

9.23g in 1000ml distilled water

Substrate Buffer for Horse Radish Peroxidase

(Voller, 1976)

Dissolve 8mg of 5-aminosalicylic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 80ml of hot distilled water (80°C). Cool and store at 4°C. Before use, warm an aliquot to room temperature and bring pH to 6.0 with 1.0M NaOH. Add 10% of 0.05% hydrogen peroxide and use immediately.

Substrate Buffer for Alkaline Phosphatase (10% Diethanolamine Buffer)

(Voller et al, 1976)

Diethanolamine	97ml
Distilled water	800ml
Sodium Azide (NaN_3)	0.2g
Magnesium Chloride ($MgCl_2$-$6H_2O$)	0.1g

Add 1.0M HCl to give pH 9.8

Adjust volume to 1000ml with distilled water.

Store at 4°C in the dark. Before use, warm an aliquot to room temperature. Add substrate (para-nitrophenyl phosphate) to a concentration of 1mg/ml (Sigma 104 phosphate substrate; Sigma Chemical Co., St. Louis, Mo.).

B. Media

Modified CCY-Medium

(Arvidson et al, 1971)

Casamino acids (Difco)	40.00g
Yeast extract	10.00g
Sodium *B*-glycerophosphate	20.00g
Sodium lactate (50%)	10ml
Sodium phosphate dibasic (Na_2HPO_4)	0.53g
Potassium phosphate monobasic (KH_2PO_4)	0.40g
dl-Tryptophan	0.53g
l-Cystine	0.10g
Distilled water	1000ml

Vitamin Stock:

Thiamine	0.02g
Nicotinic Acid	0.04g
Distilled water	100ml

Trace Elements:

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.20g
Manganous sulfate ($MnSO_4 \cdot 4H_2O$)	0.10g
Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	0.06g
Citric acid	0.06g
Distilled water	100ml

The main portion of the medium is sterilized at 15 psi for 15 minutes. The vitamin solution is first adjusted to pH 4.5 with 0.1M HCl and then heat sterilized at 5 psi for 10 minutes. The trace elements solution is sterilized at 15 psi for 15 minutes. Before use, 1% vitamin stock and trace elements are added to the medium.